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(54) Title: USE OF mtr GENE SEQUENCES FOR EXPRESSION OF FOREIGN GENES

(57) Abstract

Expression systems useful in recombinant hosts are provided which permit selection of homokaryotic recombinants capable of producing a desired foreign gene. The expression system contains its own selectable marker. A first nucleotide sequence encoding the desired foreign gene is included in an expression system containing a second nucleotide sequence derived in an illustrative embodiment from the *mtr* gene of *N. crassa* which is disposed with respect to the first sequence so as to effect homologous recombination of both the first and second sequences into the *mtr* locus of the host cell, thus disrupting the endogenous locus. Since the functional product of the *mtr* gene is required in order to make the cells susceptible to certain poisonous substances, homokaryotic recombinants are resistant to these substances. Successful recombinants can therefore be efficiently selected. The inserted expression system is capable of effecting the production of the desired protein.

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USE OF mtr GENE SEQUENCES FOR EXPRESSION OF FOREIGN GENES

This invention relates to the use of cloned sequences of the mtr gene of Neurospora crassa to express DNA encoding heterologous proteins, and to use of recessive traits to assure homokaryotic transformation in polynucleated cells.

BACKGROUND OF THE INVENTION

The translocation of aliphatic and aromatic amino acids across the plasma membrane of the ascomycete, Neurospora crassa, requires a functional gene product of the genomic mtr locus. Perkins, D.D. et al., Microbiol Rev (1982) 46:426-570. Mutations at this locus result in defective transport of neutral aliphatic and aromatic amino acids and the fungus exhibits altered cell-surface glycoproteins. These mutations can be selected by resistance to 4-methyltryptophan (4-MT) and p-fluorophenylalanine (pFPA). These substances are poisons, which cannot be transported into the cell in the absence of the mtr gene product.

The mtr locus has been mapped to the right arm of linkage group IV by Stadler, H., Genetics (1966) 54:677-685. Intralocus recombination of allelic mutants in the mtr locus has been shown by Larimer, F.W. and DeBusk, A.G., J Bacteriol (1977) 129:1636-1638.

A restriction map of a 2.9 kb fragment of genomic DNA encompassing the mtr coding region has been produced from a cosmid library of genomic DNA of Neurospora crassa by Stuart, W.D., et al., Genome (1988) 30:198-203. In a later paper, Koo, K. and Stuart, W.D., Genome (1991) 34:644-651, the complete nucleotide sequence of the open reading frame and the upstream and downstream regulatory sequences of the mtr locus of an N. crassa strain were described, along with the location of useful restriction sites. The

disclosure of these articles is incorporated herein by reference.

The 1991 article contains two minor errors in the DNA sequence there provided. They are in the upstream region of what is reported as the open reading frame. Correction of these errors results in extension of the open reading frame further into the 5' region; however, this change does not materially affect the utilization of this gene in the invention described herein. The disclosure of the parent application herein, U.S. serial no. 07/899,689 filed 17 June 1992 is also incorporated herein by reference.

Other transport related genes that confer susceptibility to certain amino acid analogs in N. crassa are also known, such as the pmb locus, which confers canavanine susceptibility.

The present invention provides materials and methods to take advantage of the necessity for a functional mtr gene product to confer susceptibility to certain metabolic poisons such as 4-MT and pFPA. By using as host a cell which is capable of providing functional mtr gene products, successful homologous recombination of an expression system for a desired protein into all functional mtr loci present in the host can be selected for directly.

DISCLOSURE OF THE INVENTION

The invention provides materials and methods for efficient transformation and selection of hosts that provide functional mtr or other recessive gene products, especially filamentous fungal hosts, which are modified to contain an expression system for a desired recombinant foreign protein. Expression systems are used for transformation which are simultaneously capable of producing the desired protein and providing a selectable marker for successful homokaryotic transformants. The expression systems are designed so as to effect homologous

recombination into native mtr loci or analogous loci in the host.

Thus, in one aspect, the invention is directed to an expression system for a gene encoding a desired protein. The expression system consists of a DNA molecule which comprises a first nucleotide sequence encoding the desired protein and a second nucleotide sequence derived from a gene that confers a recessive susceptibility to a poison, such as the mtr gene of N. crassa. The first and second sequences are disposed in relationship so as to permit homologous recombination of the first and second sequences into the target locus of the host in such a way as to incapacitate normal expression of the locus. This prevents the production of functional protein from that locus. The first, coding, nucleotide sequence is operably linked to control sequences which effect expression of the coding sequence when contained in a recombinant host cell. When all functional native, recessive loci are destroyed, the transformed host can be selected by growth on a medium containing a substrate which is poisonous to the cell requiring the gene product for transport. This general method for selecting homokaryotic transformed cells is illustrated herein using the mtr locus of N. crassa.

In other aspects, the invention is directed to filamentous fungal cells modified to contain the expression system of the invention, to methods to obtain such modified cells, and to methods to produce the desired protein using the modified cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide sequence of an approximately 2.9 kb fragment of the N. crassa mtr gene containing the entire open reading frame as well as the promoter and transcription terminating signals.

Figure 2 shows nucelotide and deduced amino acid sequences of the open reading frame of the mtr locus.

MODES OF CARRYING OUT THE INVENTION

The present invention provides, in a preferred embodiment, the relevant DNA of a wild type Neurospora crassa mtr gene locus. Figure 1 shows the complete nucleotide sequence of this wild type mtr; an approximately 2.9 kb segment of DNA derived from a cosmid library of N. crassa FGSC No. 3043 is depicted. Because of considerable homology among various strains of N. crassa and across various filamentous fungi, both allelic variants of the nucleotide sequence in Figure 1 and variants derived from alternative strains of filamentous fungal species and other organisms which exhibit unicellular growth may also be used in the invention. Provision of the sequence information of Figure 1 is adequate to permit retrieval of these alternative mtr genes. Modifications of the depicted sequence can be made by site-directed mutagenesis and portions or all of this or other retrieved sequences may be synthesized using well known solid phase nucleotide synthesis techniques. Alternate sequences can also be derived from cosmid libraries prepared from other fungi in a manner analogous to that described for the retrieval of the gene disclosed herein by Koo, K. et al., Genome (1991) 34:644-651, cited above.

Thus, as used herein, the phrase "a nucleotide sequence derived from the mtr gene of N. crassa" refers to a nucleotide sequence as shown in Figure 1, or a functional portion thereof, or a modified form thereof which contains sufficient homology to the sequence depicted to be functional in effecting homologous recombination with a native mtr locus, allelic variants thereof, corresponding loci from other organisms which are retrievable using the depicted sequence as a probe or using relevant portions of the sequence shown in Figure 1 as primers for PCR cloning. Functional portions of all of the foregoing may be used. These nucleotide sequences may be obtained by cloning the retrieved genes, modifying

retrieved genes and amplifying them, or partially or completely synthesized using standard polynucleotide synthesis techniques.

The expression systems of the present invention contain, in addition to a nucleotide sequence derived from the mtr gene of N. crassa, a gene encoding a desired protein to be produced recombinantly in a host cell. Any desired protein can be produced using the expression systems of the invention. Such proteins include both plant and animal proteins and proteins derived from microorganisms. Thus, genes encoding enzymes such as thrombin, tissue plasminogen activator, streptokinase, urokinase, alcohol dehydrogenase, various proteases such as trypsin, chymosin, enterokinase, and the like are suitable for production using the expression systems of the invention. Such enzymes may be useful as pharmaceuticals, as reagents, or as industrial enzymes. Other exemplary types of proteins whose production may be desired include growth factors and cytokines such as interleukin-2, bovine growth hormone, human growth hormone, fibroblast growth factor, TGF-α, TGF-β, bone morphogenic protein, IL-3, IL-6, and the like. In addition, the expression system may be used for the recombinant production of antibodies or fragments thereof, including chimeric antibodies as well as hormones such as insulin, ACTH, the gonadotropins, and the like. Any protein for which a practical means of production is desired and the coding sequence for which is available can be employed in the expression systems and methods of the invention.

The host cells which are useful in the invention method for production of recombinant protein using the expression systems containing nucleotide sequences derived from the mtr gene of N. crassa are cells containing genes encoding functional mtr products which confer susceptibility on the native host to substances toxic to the cells when internalized using the mtr system, such as the 4-MT

and pFPA described above. The host cells can be derived from any species, provided the relevant gene is present in these cells, and provided the cells can be grown in culture. Filamentous fungal host cells are preferred. N. crassa host cells are particularly preferred.

Cells subject to the general method described herein (selection for homokaryotic recombinants by effecting destruction of an endogenous recessive gene conferring susceptibility to a poison) may be any cells which contain such recessive genes. In a manner analogous to that described herein using a nucleotide sequence derived from the mtr locus, sequences derived from the relevant recessive gene locus are used to effect homologous recombination of a desired DNA into the genome of the host cell. The recombination results in destruction of the functionality of the native gene and thus converts the successful homokaryotic transformant into a cell resistant to the poison. Only homokaryotic transformants will therefore grow on media containing the poison.

Other recessive genes of this type, besides the mtr locus, are known. For example, N. crassa also contains a locus encoding a "pmb" product which effects the transport of basic amino acids into the cells. Canavanine, a basic substance which is toxic to native N. crassa, is then not toxic to cells with each such locus destroyed. Successful transformants can then be identified by successful growth on a medium containing canavanine.

The method disclosed herein for efficient selection of successful homokaryotic transformed cells is particularly useful where the cell to be transformed has more than a single copy of a gene locus. Thus, this approach is useful in transformation of diploid or polyploid cells, or, especially, in polynucleated cells such as those found particularly in filamentous fungal species. Since all

of the native loci must be destroyed in order to render the cells resistant, the selection method automatically provides homokaryotic transformants.

Unless otherwise stated, the present invention employs molecular biology, microbiology and recombinant DNA techniques as described, for example, Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual" (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover ed. 1985); "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" (B.D. Hames & S.J. Higgins eds. 1985); "Transcription And Translation" (B.D. Hames & S.J. Higgins eds. 1984); "Animal Cell Culture" (R.I. Freshney ed. 1986); "Immobilized Cells And Enzymes" (IRL Press, 1986); B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

The expression systems of the invention provide for insertion of heterologous DNA into cells (including spheroplasts or protoplasts) that can be multinuclear, so that a selection of homokaryotic transformants is facilitated. Homokaryotic transformants may be selected in one step from a colony or mixture containing multinuclear cells. This is particularly advantageous in dealing with Neurospora or other filamentous fungi having vegetative spores (conidia) which contain one or more nuclei. The transformation of such spores, after digestion of the cell walls to produce spheroplasts, is a random event. If the spheroplasts are multinueleated, the transforming DNA may be inserted into less than all of the nuclei that are present. Other multinucleate cells can also be used as subjects for the invention.

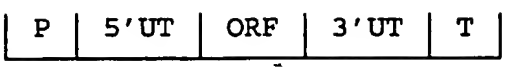
Transformation selection protocols directed to a dominant phenotype, such as the restoration of an enzyme which confers the ability to grow on minimal medium, do not select for homokaryotic transformants. Transformation of any single nucleus in any spheroplast will result in growth of transformed

colonies on selective media, even though many of the transformants will also carry untransformed nuclei in a number equal to, or in excess of, the desired transformed nucleus. In the invention method, when a recessive allele is inserted, the recessive genotype can be expressed phenotypically (and thus selected) only if it is the only allele in the culture of transformed strains, thus ensuring that selected transformants will carry only the nuclei which have the constructed gene of interest.

The illustrative expression system herein provides such a recessive allele. Neurospora cells that include wild-type mtr cannot grow on media containing 4-MT or pFPA because the transport system in the host of which the mtr gene product is a part brings these substances into the cell and the cell dies. If the host cells are made homokaryotic mtr negative mutants, the transport system is inoperative so the cell can survive on the media containing these substances, because it is not transported into the cell.

For colonies of multinucleated spheroplasts, transformation of one nucleus to mtr^- by insertion of a vector according to the present invention would still result in selection against that transformant because the nontransformed nuclei would continue to produce the mtr protein, the cell will continue to transport the poison and would therefore die as a result. Only if a transformed spheroplast has all mtr loci disrupted will it be able to resist the poison, survive the selection and grow into a strain where all the nuclei contain the inserted gene. Thus, in selecting for resistant transformants, selection of homokaryotes is assured.

The general features for use of the expression systems of the invention can be understood in terms of the diagram showing the features of the mtr gene (common features with genes in general).



The 5' untranslated region (5'UT) transcribed into the mRNA contains features which facilitate translation of the mRNA. This is followed by the open reading frame (ORF) which contains the coding region which is ultimately translated into the protein. In the mtr gene illustrated, the ORF also contains a 59-nucleotide intron which remains in the mRNA but is not translated. (See Figure 2.) Presumably, the intron contributes to enhanced stability of the mRNA. The transcribed region is completed by the 3' untranslated region (3'UT) which contains the polyadenylation signal also contributing to mRNA stability and facilitating translation.

In the DNA, the transcribed region is preceded by the promoter (P) and followed by the transcription terminating sequences (T). The transcription terminating sequences are believed to render expression more efficient, but do not appear to be necessary to effect at least some expression.

The expression systems of the invention contain some or all of the mtr sequence just discussed, or the corresponding portions of other recessive loci, and thus provide for the insertion of DNA comprising the sequence encoding the desired protein to be inserted somewhere in the overall gene or contiguous with one of its termini, provided sufficient deletion or interruption is made in the gene sequences so as to disrupt expression of the wild type gene when homologous recombination occurs.

While homologous recombination can be effected by extension at only one terminus of the desired DNA to be inserted, insertion is more efficient when the DNA to be inserted is bracketed by homologous sequences. The efficiency of homologous recombination is increased with increasing length of nucleotide sequence bearing homology to the target for recombination. It is believed that a minimum of ten

nucleotides is required, but preferably 20, more preferably 100, still more preferably 200 and still more preferably 1000 nucleotides can be included in the construct to effect recombination. The length of the nucleotide sequence provided is arbitrary; the frequency of recombination is simply increased as the length of homologous sequence increases. Furthermore, the homologous sequences, such as the mtr sequences, must be modified in some way so as to make them nonfunctional if they are to be simply ligated to one end of the inserted sequence, rather than automatically disrupted by the DNA to be inserted. Thus, expression vectors which result from the insertion into the homologous sequence of the DNA comprising the gene encoding the desired protein are preferred.

It is, of course, not necessary to utilize the complete mtr gene sequences illustrated; insertion can be made into the mtr gene in which deletions have been made. For example, substantial portions of the open reading frame of the mtr gene may be removed prior to (or after) insertion of the desired DNA.

Using the above diagram for mtr as an illustration, the position for insertion of the DNA comprising the desired coding DNA is arbitrary, but will depend on the nature of the DNA to be inserted. If the inserted DNA consists essentially of the desired coding sequence, to be placed under control of the mtr promoter, the insert must be made so that the coding sequence is in operable linkage with the mtr promoter. Similar comments apply to utilization of the mtr transcription terminator. Thus, the insert is made into the transcribed region of the mtr gene, preferably into the open reading frame. It is possible, but not necessary, to provide for the production of a fusion protein by ligating the desired coding region in open reading frame with the mtr ORF. Alternatively, the DNA comprising the desired coding sequence can be inserted under the control of its own

promoter and/or transcription terminator which, themselves, may either be homologous with the coding sequence or heterologous to it. The promoter must, of course, be functional in the host. An additional copy of the mtr promoter could also, of course, be used. Similarly, DNAs comprising coding sequences for two or more desired heterologous proteins can be inserted, some or all under control of the promoter and/or transcription terminating sequences in the mtr sequence and some or all under control of their own transcription control sequences.

The functional features of the expression system in effecting homologous recombination can be illustrated in the diagrams below which show but two of many options for the construction and function of such vectors:

In the first illustration, the vector contains the transcribed portion of a mammalian gene as a replacement for the transcribed portion of the mtr gene. Upon homologous recombination, the transcribed sequences of the mammalian gene are integrated into allele replacing the corresponding regions of the mtr gene and thus disrupting its function. This is shown diagrammatically below:

vector: ---PPPP-MAMMALIAN GENE-TTTTTT----

integrates into

genome: ---PPPPP-MTR GENE-TTTTTT------

at the mtr locus disrupting the mtr gene and producing the allele:

---PPPPP-MAMMALIAN GENE-TTTTTT---.

In a second alternative, multiple genes under control of their own promoters and terminators can be inserted between the controls of the host mtr gene as shown in the following diagram:

vector:---PPPPP-GENEt-pGENEt-pGENEt-pGENEt-TTTTT

integrates into

genome: ---PPPPP-MTR GENE-TTTTTT------

at the mtr locus disrupting the mtr gene and producing the allele:

---PPPPP-GENEt-pGENEt-pGENEt-pGENEt-TTTTTT---.

In this illustration, only the first gene in the sequence is under control of the mtr promoter; the remaining genes bear their own control sequences. Again, the function of the wild type mtr gene will be destroyed.

Of course, substantial portions of the open reading frame of the mtr gene can also be retained, if desired, so long as insufficient sequences are provided to effect production of a functional protein gene product. Indeed, deletions, additions and alterations in the sequence encoding the mtr protein product may be made in the expression system constructed so long as the remaining sequences fail, when integrated, to provide a functional product, but are functional to effect integration.

Thus, the mtr gene sequences contained in the expression vector have at least two functions:

(1) they effect homologous integration of the inserted or contiguous DNA comprising the coding sequence for the desired protein, and

(2) they are of such a nature that when homologous combination occurs, no functional mtr protein is produced. Thus, they cannot themselves be sufficient to restore mtr protein function.

The construction of expression systems of the invention are illustrated by the initial construction of a host vector, designated herein pXpress, which includes the mtr gene sequence shown in

Figure 1. This vector is suitable for amplification in E. coli, and provides endogenous insertion sites for the DNA containing the desired coding sequence. Also illustrative of host vectors is pDXpress, an analogous vector which is identical to pXpress except that an additional polylinker in the original host vector, from which pXpress was constructed, is deleted.

The illustrative vector pXpress is constructed from the vector pBN3 which is described in Stuart, W.D., et al., Genome (1988) 30:198-203. pBN3 contains the 2783 bp N. crassa genomic DNA containing the mtr gene shown in Figure 1, which is bracketed by a BglII site in the gene and a BamHI site contained in the vector. pBN3 was digested with BamHI and BglII and the segment containing the mtr gene was inserted into the BamHI site of the commercially available vector, pTZ18R, obtained from Pharmacia. This produced clones pN807 and pN816 wherein the EcoRI site contained in the polylinker of the pTZ18R vector is upstream of the ORF; clones in the opposite orientation were designated pN846 and pN839. pXpress is a version of pN846 wherein the 5' polylinker of pTZ18R is deleted. The pXpress vector has useful cloning sites for insertion of the desired DNA in the upstream region just downstream of the mtr promoter (SalI/AccI/HincII) (position 307 in Figure 1) and also in the latter third of the ORF (HincII at position 1406 and AccI at position 1920).

Thus, in one illustrative embodiment, pXpress may be used directly as an insertion vector to construct the expression system of the invention by cleaving with AccI, which deletes most of the open reading frame while retaining both the promoter and transcription terminator of the mtr gene. In another embodiment, the vector may first be digested with SalI, which recognizes only the upstream post-promoter site, and then with HincII which, since the upstream recognition sites overlap, now recognizes only the

downstream site to produce a SalI-blunt opening into which the desired DNA may be inserted in a directionally controlled way.

pDXpress is a modified form of pXpress in which the linker provided in the host vector pTZ18R at the 3' end of the pN846 insert is deleted, thus eliminating additional restriction sites duplicate to those available in the sequence of the mtr gene.

After construction of a suitable expression vector by insertion of the desired coding sequence into the host vector containing the nucleotide sequence derived from mtr, cells of the desired recombinant host are treated with the vector under conditions which favor the uptake of the vector DNA into the cells. These transformation conditions are standard and depend on the nature of the cells to be transformed. For transformation of filamentous fungi, the preferred hosts of the invention, spheroplasts or protoplasts are generally used and the vector is linearlized prior to treating the cells. The transformants are then selected on medium containing a substance to which mtr confers susceptibility, such as pFPA or 4-MT. Successful homokaryotic transformants can survive in this medium and can be cloned.

The cloned cells lines are then used in culture for production of recombinant protein encoded by the inserted DNA. The protein thus produced may be contained intracellularly or secreted into the medium, depending on the expression system construction used, as is understood in the art. After culturing under conditions which encourage the production of the protein, the protein produced is recovered from the culture and, if desired, purified using standard techniques known in the art. Alternatively, the cultures themselves or the culture media may provide the protein in sufficient purity for its intended use, as may be the case, for example, for the production of industrial enzymes or enzymes used for organic synthesis steps. Of course, if the protein produced

is intended for pharmaceutical use, it may be desirable to purify the protein produced to a suitable level for formulation.

The following examples illustrate, but do not limit the invention.

Example 1

Preparation of pBN3

11.3 μg of BglII digested 5:4H DNA (DNA from cosmid 5:4H in the Vollmer-Yanosky library, described in Vollmer et al., PNAS (1986) 83:4869-4873), was electrophoresed in a preparative 1% LMT-agarose gel. 15.0 μl (70 ng of DNA) of the liquified agarose gel, containing the 15.0 kbp fragment was mixed with 1.5 μl H_2O, 2.0 μl 10X T4 ligase buffer at 37°C and maintained at this temperature for 15 min. The cosmid 5:4H is capable of transforming an mtr negative host strain, as are 5:3B and 5:10H from the same library. Then 1.5 μl 1.4 U/μl T4 DNA ligase was added. The contents were briefly mixed by flicking the tube and brought down with a quick pulse in a microcentrifuge. The reaction was incubated at 15°C overnight.

At the end of the incubation, 150 μl Tris. Cl/$CaCl_2$ was added to the ligation reaction which was heated to 65-70°C for 5 minutes to liquify the agarose. The condensation was brought down with a rapid pulse, and the tube was immersed into an ice bath. The contents, after sufficient cooling, were dispensed into a microfuge tube containing 200 μl competent E. coli LE392 cells. The transformation and plating of the cell/media mix was the same as previously described.

Example 2

Generation of pN807 and pN846

6 μg pTZ18R DNA as digested to completion with 17 U BamHI and the final concentration adjusted to 60 ng/μl. 12.0 μg pBN3 DNA was double digested with 36 U BglII and 34 U BamHI. The sample was

electrophoresed in a 1% LMT-agarose gel and the 2.9 kbp fragment was excised. The agarose plug was immersed in H_2O at room temperature (60 min.) to remove excess EtBr and 1X TBE buffer. The agarose plug, containing 600 ng of the 2.9 kbp fragment was liquified by heating and mixed with 120 ng of BamHI digested pTZ18R vector. 5X T4 ligase buffer and 2 U T4 DNA ligase was mixed into the solution at 37°C for a final volume of 90 μl. The reaction was incubated at room temperature (overnight).

A half volume aliquot of the reaction mix was liquified and mixed with an equal volume of Tris Cl/$CaCl_2$ solution, and cooled in an ice bath. The solution was used to transform 200 μl competent E. coli NM522 cells. The transformed cells were spread on X-gal indicator plates to detect recombinant DNA molecules. The numbered clones pN389 and pN846 are the same clones picked and numbered independently from different colonies. The numbered clones pN807 and pN816 are the same clones picked and numbered independently from different colonies, and contain the pBN3 insert in opposite orientation from that of pN839/846?

Example 3

Preparation of pXpress and pDXpress

Five micrograms of pN846 DNA were isolated from E. coli NM522 by standard methods (Koo and Stuart, 1991). The DNA was double digested with XbaI and HindIII, treated with Klenow and NTPs, cleaned with Geneclean (Bio 101), and ligated with 400 units of DNA T4 polymerase at room temperature overnight. The ligation mixture was used to transform E. coli NM522 host cells and selected for Amp^r. Transformed colonies were picked and grown in 1.5 ml liquid cultures in tubes overnight. Plasmid DNA was isolated and tested for the presence of HindIII, XbaI and PstI restriction sites. Isolates which had lost the three sites were then tested for the remaining sites

expected to be in pN846. One plasmid which had lost the expected sites and retained the expected sites was designated plasmid pXpress.

To prepare pDXpress, pXpress was digested with BamHI and EcoRI, the ends polished with Klenow, and the DNA is relegated (in the presence of SmaI, to reduce the number of copies of partially cut and relegated vector). The ligation mixture is transformed into E. coli host cells and transformants selected for resistance to ampicillin. Plasmid DNA from resistant colonies is tested for loss of EcoRI, BamHI and SacI sites. A plasmid meeting these criteria and also retaining the internal KpnI sites of pXpress was designated pDXpress (plasmid downstream express).

Example 4

Production of Chymosin

The gene encoding bovine chymosin (Moir et al., Gene (1982) 29:127-138) was inserted into pXpress as a XhoI/HindIII-blunted fragment. pXpress was digested with SalI and then with HincII, and blunted. This deletes the portion of the mtr gene between positions 307 and 1406 shown in Figure 1.

A sample of 0.5 μg of the chymosin open reading frame was ligated into 0.5 μg of the cleaved vector using 40 units of T4 ligase incubated overnight at 16°C. Some fragments ligate SalI to XhoI and blunt HindIII to blunt HincII (losing all four restriction sites).

The ligated fragments were transformed into competent E. coli cells DH-5alpha and selected for Amp^r. Plasmids were isolated and digested with EcoRV to confirm the insert. Plasmids containing the insert in the correct orientation were renamed pNEC for plasmid neutral (mtr) expression of chymosin.

Transformation of pNEC into Neurospora spheroplasts was accomplished by standard methods. See Koo and Stuart (1991) supra. pNEC DNA was

linearized by cutting with SacI and 5 μg of linearized pNEC was used to transform $1^{-}X\ 10^{8}$ spheroplasts of strain 74a "wild" type Neurospora (Fungal Genetics Stock Center, Dept. of Microbiology, University of Kansas Medical Center, Kansas City, Kansas 66103). The mixture was taken up in 15 ml of minimal top agar and spread onto a bottom plate containing 0.05 mg/ml pFPA. Plates were screened 3 days later. Eighteen colonies were picked and grown on solid Vogel's 1X media containing 0.05 mg/ml pFPA in tube slants.

Colonies were transformed to liquid cultures of 1X Vogel's with 2% sucrose in double distilled H_2O. The liquid was collected and assayed for chymosin activity using 25 μl culture, 125 μl 10 mM Phosphate Buffered Sodium (PBS), and 150 μl clotting buffer (4.5 mg/ml $CaCl_2$, 3.0 mg/ml NaOAc in 10 mg/ml nonfat milk). Samples were tested on a 64 well microtiter plate, incubated at 37°C for 15 min then assayed by absorbance at 630 nm on a Dynatech MR 5000 ELISA machine. Among the 18 transformants, four showed milk clotting ability. Chymosin concentrations ranging from 1.6-4.6 μg/ml were extrapolated from known chymosin standards.

ELISA assay was performed on the pNEC transformants using rabbit antibovine chymosin antibody. Secondary antibody, goat antirabbit Ab conjugated to alkaline phosphatase was used to visualize chymosin concentrations. The ELISA reactions indicated chymosin concentrations ranging from 1-16 μg/ml. These results indicated that not all of the chymosin product was biologically active. Western blots of PAGE gels showed that some of the chymosin in the Neurospora media was in the form of prochymosin which is inactive until converted by incubation at pH4 for several hours.

Example 5

Expression of Relaxin Gene

A gene coding for the open reading frame of porcine relaxin (Haley et al., DNA (1982) 1:155-162) was inserted into the pXpress in a manner similar to the protocols used for chymosin. The gene was copied using the PCR reaction using primers designed so that a KpnI site and the Neurospora translation consensus sequence (Koo and Stuart 1991) were added to the 5' end of the gene and a BamHI site was added to the 3' end. The PCR product was polished with T4 polymerase and inserted in the plasmid pXpress at the HincII sites in a manner identical to the chymosin construction.

Orientation was confirmed by double digestion with EcoRV and BamHI and with single digestion with KpnI. Inserts with 5' end of the gene following the mtr promoter were used to transform Neurospora spheroplasts as in Example 4. Transformants resistant to pFPA were transferred to liquid cultures and the media screened for production of relaxin by ELISA assay using a rabbit anti-relaxin antibody and goat anti-rabbit secondary antibody. Six transformants secreted a protein which cross reacted with relaxin antibody, but at levels 10 to 100 times lower than chymosin as determined by ELISA.

CLAIMS

1. An expression system for expressing a gene encoding a desired protein in a recombinant host having a functional mtr locus, said expression system consisting of a DNA molecule which molecule comprises:

a first nucleotide sequence encoding said desired protein;

a second nucleotide sequence derived from the mtr gene of *N. crassa* disposed in relationship to said first sequence so as to permit homologous recombination of said first and second sequences into the mtr locus of said host so as to incapacitate the mtr locus in said host from effecting production of functional mtr protein;

wherein said first nucleotide sequence is operably linked to control sequences which effect expression of said encoding sequence, when contained in the recombinant host cell.

2. The expression system of claim 1 wherein at least a portion of said second sequence is disposed at the 5′ terminus of the said first nucleotide sequence.

3. The expression system of claim 2 wherein said portion comprises the mtr promoter operably linked to said first nucleotide sequence.

4. The expression system of claim 1 wherein at least a portion of said second nucleotide sequence is disposed at the 3′ terminus of said first nucleotide sequence.

5. The expression system of claim 4 wherein said portion comprises the mtr transcription terminator sequences operably linked to said first nucleotide sequence.

6. The expression system of claim 1 wherein said first nucleotide sequence is operably linked to a promoter homologous to said first nucleotide sequence, or wherein said first DNA sequence is operably linked to a promoter which is heterologous both to said first and second nucleotide sequences, or

wherein said first nucleotide sequence is operably linked to a transcription terminator homologous to said first nucleotide sequence, or

wherein said first DNA sequence is operably linked to a transcription terminator heterologous to both said first and second nucleotide sequences, or

wherein said first nucleotide sequence is an open reading frame with the coding region of said second sequence, or

wherein said second nucleotide sequence comprises less than the complete sequence of the mtr gene.

7. A recombinant host cell which contains the expression system of claim 1.

8. The cell of claim 7 which is Neurospora crassa.

9. A method to produce a desired protein which method comprises culturing the host cell of claim 7 under conditions wherein expression of the nucleotide sequence encoding the desired protein is effected to as to produce said protein in the culture; and

recovering the protein from the culture.

10. The method of claim 9 wherein said desired protein is relaxin or chymosin.

11. A method to select for a recombinant host cell which has, integrated into all mtr loci in

said cell, the expression system of claim 1, which method comprises culturing said candidate cells on a medium containing a substance which is lethal to said cell in the presence of a functional mtr gene product; and

selecting cells capable of growth on said medium.

12. A method to obtain cells which are homokaryotic transformants with a desired DNA, which method comprises contacting said host cells,

in a form susceptible for uptake of DNA molecules and under conditions which are suitable for the uptake of DNA molecules,

with a DNA molecule which comprises said desired DNA contiguous to a sequence homologous to a recessive gene that confers susceptibility to a poison, so as to permit homologous recombination of said DNA molecule into the locus of said recessive gene to prevent expression of said gene;

culturing the resulting cells in a medium containing said poison; and

recovering cells which are capable of growth in the presence of said poison.

13. A method to obtain cells which are homokaryotic transformants with a desired DNA, which method comprises

culturing cells that have been treated, in a form susceptible for uptake of DNA and under conditions which are suitable for the uptake of DNA molecules with a DNA molecule which comprises said desired DNA contiguous to a sequence homologous to a recessive gene that confers susceptibility to a poison, so as to permit homologous recombination of said DNA molecule into the locus of said recessive gene to prevent expression of said gene;

in a medium containing said poison; and

recovering cells which are capable of growth in the presence of said poison.

```
### DNA Strider 1.0 ### Tuesday, June 15, 1993  10:07:28 AM

New 2.9 kb DNA seqn -> List

DNA sequence  2783 b.p.    AGATCCGCCTCG ... GATGTTCCAGGC  linear

         |   10      |   20      |   30      |   40      |   50      |   60
   1 AGATCCGCCT CGCCCCAAGC GCATCCCAAC GCGGCGTGCT TATATGTCGC TCTTCCCTCT 60
  61 CACGTACCTC GCAAGTACCT GTCTCATCTG GCACCCGCCT TCTCCATCCC TCTTCTTCAC 120
 121 TACTTAATCT GCCCCGGTCC CCCGCAGTTC ATCCTGTCTC TCAGACCTTG GATCGTCTGT 180
 181 TGGTCTTTTC GCTTATTGTC CGTACCCCCA TCCATATTTA TTCCTGCCTG GGCCCCCAGA 240
 241 CAGCATTTTC TCTTTCCCTC CCTCTTTAGC CATCCAAACA GCTTGAGAAG CGAAAATCAA 300
 301 GCCACTGTCG ACAAAGGCCT TCAACAAGGC CTCTTTACCA CCCAACATGG ACTCGCAATA 360
 361 CGAGACAAAA AAGAATGACC CAAACGCCAT CATGCCGTAC CCAGAGTCAA ACGATGAGCA 420
 421 TGTTGGCGAG GTCCGCGGCT TGGGCGGCCC CATCATGGAC AAGGAGCCTG AGGCCCAGGA 480
 481 GGGCCATGCC AAGTTCCACC GTCTCGGCTG GAAACGTCTG ACGGTCGTCC TCATCGTCGA 540
 541 GGCCATTGCC CTCGGCTCTC TCTCGCTTCC CGGCGCCTTC GCTACCCTTG GCATGGTGCC 600
 601 TGGTGTTATT CTCTCTGTCG GCATGGGACT CATCTGCATC TACACGGCTC ACGTTATCGG 660
 661 ACAAACCAAG CTCAAGCACC CTGAAATCGC CCACTATGCC GACGTTGGTC GTGTCATGTT 720
 721 TGGAAGATGG GGATATGAAA TCATCAGCTT CATGTTTGTT CTGCAACTGA TCTTCATCGT 780
 781 CGGCTCCCAC GTCCTCACTG GCACCATCAT GTGGGGCACC ATCACGGATA ACGGCAACGG 840
 841 TACCTGCTCT CTCGTCTTCG GCATTGTCTC CGCCATCATT CTCTTCCTCC TTGCCATTCC 900
 901 TCCCAGTTTC GCCGAGGTTG CCATCCTTGG ATACATCGAT TTCGTCTCCA TCTGCGCCGC 960
 961 CATCCTCATC ACCATGATTG CTACTGGCAT TCGCTCGAGC CACCAGGAGG GTGGTCTCGC 1020
1021 TGCTGTTCCC TGGTCTTGCT GGCCCAAGGA GGACCTTAGC CTTGCTGAGG GCTTCATTGC 1080
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FIG.1A

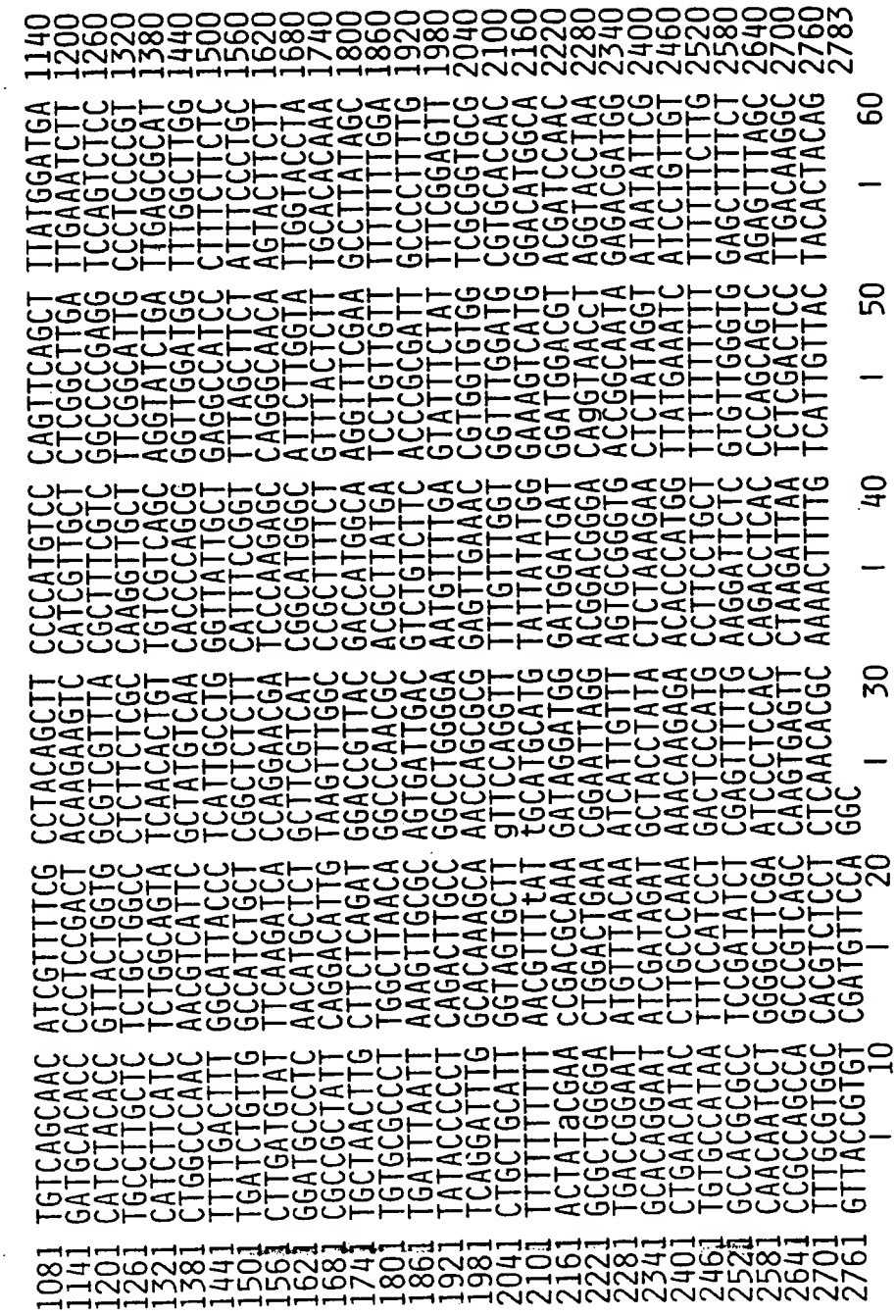
1081 TGTCAGCAAC ATCGTTTTCG CCTACAGCTT CCCCATGTCC CAGTTCAGCT TTATGGATGA 1140
1141 GATGCACACC CCCTCCGACT ACAAGAAGTC CATCGTTGCT CTCGGCTTGA TTGAAATCTT 1200
1201 CATCTACACC GTTACTGGTG GCGTCGTTTA CGCTTTCGTC GGCCCCGAGG TCCAGTCTCC 1260
1261 TGCCTTGCTC TCTGCTGGCC CTCTTCTCGC CAAGGTTGCT TTCGGCATTG CCCTCCCCGT 1320
1321 CATCTTCATC TCTGGCAGTA TCAACACTGT TGTCGTCAGC AGGTATCTGA TTGAGCGCAT 1380
1381 CTGGCCCAAC AACGTCATTC GCTATGTCAA CACCCCAGCG GGTTGGATGG TTTGGCTTGG 1440
1441 TTTTGACTTT GGCATTACCC TCATTGCCTG GGTTATTGCT GAGGCCATCC CTTTCTTCTC 1500
1501 TGATCTGTTG GCCATCTGCT CGGCTCTCTT CATTTCCGGT TTTAGCTTCT ATTTCCCTGC 1560
1561 CTTGATGTAT TTCAAGATCA CCAGGAACGA TCCCAAGAGC CAGGGCAACA AGTACTTCTT 1620
1621 GGATGCCCTC AACATGCTCT GCTTCGTCAT CGGCATGGGC ATTCTTGGTA TTGGTACCTA 1680
1681 CGCCGCTATT CAGGACATTG TAAGTTTGGC CCGCTTTTCT GTTTACTCTT TGCACACAAA 1740
1741 TGCTAACTTG CTTCTCAGAT GGACCGTTAC GACCATGGCA AGGTTTCGAA GCCTTATAGC 1800
1801 TGTGCGCCCT TGGCTTAACA GGCCCAACGC ACGCTTATGA TCCTGTTGTT TTTTTTTGGA 1860
1861 TGATTTAATT AAAGTTGCGC AGTGATTGAC GTCTGTCTTC ACCCGCGATT GCCCCTTTTG 1920
1921 TATACCCCCT CAGACTTGCC GGCCTGGGGA AATGTTTTGA GTATTTCTAT TTTCGGAGTT 1980
1981 TCAGGATTTG GCACAAAGCA AACCAGCGCG GAGTTGAAAC CGTGGTGTGG TCGCGGTGCG 2040
2041 CTGCTGCATT GGTAGTGCTT gTTCCAGGTT TTTGTTTGGT GGTTTGGATG CGTGCACCAC 2100
2101 TTTTTTTTTT AACGTTTtAT tGCATGCATG TATTATATGG GAAAGTCATG GGACATGGCA 2160
2161 ACTATaCGAA CCGACGCAAA GATAGGATGG GATGGATGAT GGATGGACGT ACGATCCAAC 2220
2221 GCGCTGGGGA CTGGACTGAA CGGAATTAGG ACGGACGGGA CAgGTAACCT AGGTACCTAA 2280
2281 TGACCGGAAT ATGTTTACAA ATCATTGTTT AGTGCGGGTG ACCGGCAATA GAGACGATGG 2340
2341 GCACAGGAAT ATCGATAGAT GCTACCTATA CTCTAAAGAA CTCTATAGGT ATAATATTCG 2400
2401 CTGAACATAC CTTGCCCAAA AAACAAGAGA ACACCCATGG TTATGAAATC ATCCTGTTGT 2460
2461 TGTGCCATAA TTTCCATCCT GACTCCCATG CCTTCCTGCT TTTTTTTTTT TTTTTTCTTG 2520
2521 GCCACGCGCC TCCGATATCT CGAGTTTTTG AAGGATTCTC GTGTTGGGTG GAGCTTTTCT 2580
2581 CAACAATCCT GGGGCTTCGA ATCCCTCCAC CAGACCTCAC CCCAGCAGTC AGAGTTTAGC 2640
2641 CCGCCAGCCA GCCCGTCAGC CAAGTGAGTT CTAAGATTAA TCTCGACTCC TTGACAAGGC 2700
2701 TTTGCGTGGC CACGTCTCCT CTCAACACGC AAAACTTTTG TCATTGTTAC TACACTACAG 2760
2761 GTTACCGTGT CGATGTTCCA GGC 2783
1 10 20 30 40 50 60

FIG.1B

FIG.2A

```
New 2.9 kb DNA seqn [347 to 1699] --> 1-phase Translation
DNA sequence   2783 b.p.  AGATCCGCCTCG ... GATGTTCCAGGC  linear

347  /    1                               377  /   11
ATG GAC TCG CAA TAC GAG ACA AAA AAG AAT GAC CCA AAC GCC ATC ATG CCG TAC CCA GAG
met asp ser gln tyr glu thr lys lys asn asp pro asn ala ile met pro tyr pro glu
407  /   21                               437  /   31
TCA AAC GAT GAG CAT GTT GGC GAG GTC CGC GGC TTG GGC GGC GGC ATC ATG GAC AAG GAG
ser asn asp glu his val gly glu val arg gly leu gly gly gly ile met asp lys glu
467  /   41                               497  /   51
CCT GAG GCC CAG GAG GGC CAT GCC AAG TTC CAC CGT CTC GGC TGG AAA CGT CTG ACG GTC
pro glu ala gln glu gly his ala lys phe his arg leu gly trp lys arg leu thr val
527  /   61                               557  /   71
GTC CTC ATC GTC GAG GCC ATT GCC CTC GGC TCT CTC TCG CTT CCC GGC GCC TTC GCT ACC
val leu ile val glu ala ile ala leu gly ser leu ser leu pro gly ala phe ala thr
587  /   81                               617  /   91
CTT GGC ATG GTG CCT GGT GTT ATT CTC TCT GTC GGC ATG GGA CTC ATC TGC ATC TAC ACG
leu gly met val pro gly val ile leu ser val gly met gly leu ile cys ile tyr thr
647  /  101                               677  /  111
GCT CAC GTT ATC GGA CAA ACC AAG CTC AAG CAC CCT GAA ATC GCC CAC TAT GCC GAC GTT
ala his val ile gly gln thr lys leu lys his pro glu ile ala his tyr ala asp val
707  /  121                               737  /  131
GGT CGT GTC ATG TTT GGA AGA TGG GGA TAT GAA ATC ATC AGC TTC ATG TTT GTT CTG CAA
gly arg val met phe gly arg trp gly tyr glu ile ile ser phe met phe val leu gln
767  /  141                               797  /  151
CTG ATC TTC ATC GTC GGC TCC CAC GTC CTC ACT GGC ACC ATC ATG TGG GGC ACC ATC ACG
leu ile phe ile val gly ser his val leu thr gly thr ile met trp gly thr ile thr
827  /  161                               857  /  171
GAT AAC GGC AAC GGT ACC TGC TCT CTC GTC TTC GGC ATT GTC TCC GCC ATC ATT CTC TTC
asp asn gly asn gly thr cys ser leu val phe gly ile val ser ala ile ile leu phe
887  /  181                               917  /  191
CTC CTT GCC ATT CCT CCC AGT TTC GCC GAG GTT GCC ATC CTT GGA TAC ATC GAT TTC GTC
leu leu ala ile pro pro ser phe ala glu val ala ile leu gly tyr ile asp phe val
947  /  201                               977  /  211
TCC ATC TGC GCC GCC ATC CTC ATC ACC ATG ATT GCT ACT GGC ATT CGC TCG AGC CAC CAG
ser ile cys ala ala ile leu ile thr met ile ala thr gly ile arg ser ser his gln
1007 /  221                               1037 /  231
GAG GGT GGT CTC GCT GCT GTT CCC TGG TCT TGC TGG CCC AAG GAG GAC CTT AGC CTT GCT
glu gly gly leu ala ala val pro trp ser cys trp pro lys glu asp leu ser leu ala
1067 /  241                               1097 /  251
GAG GGC TTC ATT GCT GTC AGC AAC ATC GTT TTC GCC TAC AGC TTC GCC ATG TGC CAG TTC
glu gly phe ile ala val ser asn ile val phe ala tyr ser phe ala met cys gln phe
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1127 /  261                                  1157 /  271
AGC TTT ATG GAT GAG ATG CAC ACC CCC TCC GAC TAC AAG AAG TCC ATC GTT GCT CTC CGC
ser phe met asp glu met his thr pro ser asp tyr lys lys ser ile val ala leu gly
1187 /  281                                  1217 /  291
TTG ATT GAA ATC TTC ATC TAC ACC GTT ACT GGT GGC GTC GTT TAC GCT TTC CTC GGC CCC
leu ile glu ile phe ile tyr thr val thr gly gly val val tyr ala phe val gly pro
1247 /  301                                  1277 /  311
GAG GTC CAG TCT CCT GCC TTG CTC TCT GCT GGC CCT CTT CTC GCC AAG GTT GCT TTC GGC
glu val gln ser pro ala leu leu ser ala gly pro leu leu ala lys val ala phe gly
1307 /  321                                  1337 /  331
ATT GCC CTC CCC GTC ATC TTC ATC TCT GGC AGT ATC AAC ACT GTT GTC GTC AGC AGG TAT
ile ala leu pro val ile phe ile ser gly ser ile asn thr val val val ser arg tyr
1367 /  341                                  1397 /  351
CTG ATT GAG CGC ATC TGG CCC AAC AAC GTC ATT CGC TAT GTC AAC ACC CCA GCG GGT TGG
leu ile glu arg ile trp pro asn asn val ile arg tyr val asn thr pro ala gly trp
1427 /  361                                  1457 /  371
ATG GTT TGG CTT GGT TTT GAC TTT GGC ATT ACC CTC ATT GCC TGG GTT ATT GCT GAC GCC
met val trp leu gly phe asp phe gly ile thr leu ile ala trp val ile ala glu ala
1487 /  381                                  1517 /  391
ATC CCT TTC TTC TCT GAT CTG TTG GCC ATC TGC TCG GCT CTC TTC ATT TCC GGT TTT AGC
ile pro phe phe ser asp leu leu ala ile cys ser ala leu phe ile ser gly phe ser
1547 /  401                                  1577 /  411
TTC TAT TTC CCT GCC TTG ATG TAT TTC AAG ATC ACC AGG AAC GAT GCC AAG AGC CAG GGC
phe tyr phe pro ala leu met tyr phe lys ile thr arg asn asp ala lys ser gln gly
1607 /  421                                  1637 /  431
AAG AAG TAC TTC TTG GAT GCC CTC AAC ATG CTC TGC TTC GTC ATC GGC ATG GGC ATT CTT
lys lys tyr phe leu asp ala leu asn met leu cys phe val ile gly met gly ile leu
1667 /  441                                  1697 /  451
GGT ATT GGT ACC TAC GCC GCT ATT CAG GAC ATT GTA AGT TTG GCC CGC TTT TCT GTT TAC
gly ile gly thr tyr ala ala ile gln asp ile

1727 /                                          1759 /
TCT TTG CAC ACA AAT GCT AAC TTG CTT CTC AG ATG GAC CGT TAC GAC CAT GGC AAG GTT
                                           met asp arg tyr asp his gly lys val

    1789 /
TCC AAG CCT TAT AGC TGT GCG CCC TTG GCT TAA
ser lys pro tyr ser cys ala pro leu ala OCH
```

FIG.2B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/05866

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 5/10, 15/16, 15/80
US CL :435/69.1, 69.4, 172.3, 254.11, 254.4, 320.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 69.4, 172.3, 254.11, 254.4, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Dialog (Biosis, Medline, CAS, Biotech Abstr.)
APS
Searh terms: MTR and Neurospora

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Genome Vol. 30, No. 2, issued 1988, Stuart et al., "Cloning of mtr an amino acid transport gene of Neurospora crassa", pages 198-203, see abstract no. 6581989.	1-13
Y	Genetics Vol. 116 (1 part 2), issued 1987, Koo et al., "Cloning of the mtr gene", page 531, see abstract no. 7.6.	1-13
Y	Genome Vol. 34, No. 4, issued 1991, Koo et al., "Sequence and structure of mtr an animo acid transport gene of Neurospora crassa", pages 644-651, see abstract no. 8671122.	1-13

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 04 August 1993	Date of mailing of the international search report 26 AUG 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer JOHN LEGUAYDER Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*